Allelic variation of a *Beauveria bassiana* (Ascomycota: Hypocreales) minisatellite is independent of host range and geographic origin

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Abstract: The minisatellite locus, *BbMin1*, was isolated from a partial *Beauveria bassiana* genomic library that consisted of poly(GA) flanked inserts. Polymerase chain reaction (PCR) of the *BbMin1* repeat demonstrated allele size variation among 95 *B. bassiana* isolates. Amplification was also observed from single isolates of *Beauveria amorpha*, *Beauveria brongniartii*, and *Beauveria caledonica*. Eight alleles were identified at the haploid locus, where repeat number fluctuated between one and fourteen. AMOVA and θ (F_{st}) indicated that fixation of repeat number has not occurred within pathogenic ecotypes or geographically isolated samples of *B. bassiana*. Selective neutrality of allele size, the rate of *BbMin1* mutation, and the age of the species may contribute to host and geographic independence of the marker. Presence of alleles with a large number of repeat units may be attributed to the rare occurrence of somatic recombination or DNA replication error. The molecular genetic marker was useful for the identification of genetic types of *B. bassiana* and related species.

Key words: *Beauveria bassiana*, strain identification, minisatellite variation.

Résumé : Le minisatellite *BbMin1* a été isolé d'une banque génomique partielle du *Beauveria bassiana* composée d'inserts contenant une suite poly(GA). L'amplification PCR (réaction de polymérisation en chaîne) du locus *BbMin1* a révélé de la variation quant à la taille des allèles au sein d'une collection de 95 isolats du *B. bassiana*. L'amplification a également été obtenue chez un isolat unique du *Beauveria amorpha*, du *Beauveria brogniartii* et du *Beauveria caledonica*. Huit allèles ont été identifiés pour le locus haploïde et le monomère était répété entre une et huit fois. Des analyses AMOVA et θ (F_{st}) ont montré que la fixation du nombre de répétitions ne s'est pas produite au sein des écotypes pathogènes ou au sein d'isolats du *B. bassiana* qui montrent un isolement géographique. La neutralité de la taille des allèles et du taux de mutation de *BbMin1* sur le plan de la sélection ainsi que l'âge de l'espèce pourraient contribuer à l'absence de corrélation entre ce marqueur et l'hôte ou l'origine géographique. La présence d'allèles présentant un grand nombre de répétitions pourrait être attribuable à de rares événements de recombinaison somatique ou à des erreurs de réplication. Ce marqueur génétique s'est avéré utile pour l'identification de certains génotypes du *B. bassiana* et de d'autres espèces.

Mots clés : *Beauveria bassiana*, identification de souches, variation des minisatellites.

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Introduction

The haploid imperfect filamentous fungus *Beauveria bassiana* (Bals.) Vuill. (Ascomycota: Hypocreales) has both endophytic and entomopathogenic characteristics. Agricultural biocontrol of *Ostrinina nubilalis*, (Hübner) (Lepidoptera: Crambidae) (Bing and Lewis 1992; Bing and Lewis 1991) and *Diabrotica* spp. (Coleoptera: Chrysomelidae) (Krueger and Roberts 1997; Mulock and Chandler 2000) has been documented. Ambiguous results have been provided in regard to host specialization and geographic distribution of genetic variants of *Beauveria* spp. Specifically, Viaud et al. (1996) and Neuveglise et al. (1994) indicated that molecular variation among *Beauveria* isolates was related to insect host range using RFLP and internal transcribed spacer region analysis, respectively. Similar correlation was found based on isozyme marker data (Poprawski et al. 1989; Mugnai et al. 1989). PCR–RAPD (Williams et al. 1990; Welch and McClelland 1990) genotyping of *B. bassiana* indicated that isolates from the sugar cane borer *Diatraea saccharalis* shared ≥80% of 276 bands (Berretta et al. 1998). Regional variation in PCR–RAPD marker data of *Beauveria brongniartii* isolates from the European cockchafer, *Melolontha* spp., in France indicated that a high degree of similarity was present (Cravanzola et al. 1997; Piatti et al. 1998). Cravanzola et al. (1997) further indicated that differences between most strains represent minor variations of a

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common genotype, yet it was stated that the similarities in genotype failed to show a correlation between genotype and pathogenicity (Cravanzola et al. 1997; Piatti et al. 1998). The high degree of relatedness among *Beauveria* isolates was suggested to result from clonal propagation or recent speciation within the genus (St. Leger et al. 1992; Bidochka et al. 1994; Viaud et al. 1996).

In contrast, Urtz and Rice (1997) used PCR–RAPD analysis to distinguish two separate genetic groups of *B. bassiana* that infected the rice water weevil, *Lissorhoptrus oryzophilus*, in Louisiana that were 45% divergent at 172 polymorphic bands. Urtz and Rice (1997) also suggested that the two groups represented different populations that existed sympatrically. Based on PCR–RAPD and RFLP, Maurer et al. (1997) showed that *B. bassiana* isolates derived from coleopteran insect species showed a high level of genetic differentiation. Also, no evidence for host-range clustering was shown for the entomopathogenic fungi *Metarhizium anisopliae* and *Metarhizium flavoviride* (Bidochka et al. 1994) when PCR–RAPD bands were analyzed. More recently, microsatellite data from *Aspergillus flavus* reported a lack of significant genetic similarity of infective types (St. Leger et al. 2000). Geographic component of isolate variation was also found not to contribute to isolate differentiation. In several instances *B. bassiana* isolates from the same region, collected from the same insect species, were genetically dissimilar (Berretta et al. 1998; Urtz and Rice 1997), or similar genetic types were described from widely separated geographic locations (St. Leger et al. 1992; Bidochka et al. 1994; Poprawski et al. 1989).

Microsatellite loci are described as having two to six tandemly repeated nucleotide units, whereas minisatellites are composed of a variety of larger repeat units (Tautz 1993). Polymorphic minisatellite alleles could arise via unequal crossover (Jeffreys et al. 1985; Jeffreys et al. 1988), gene conversion (Bishop et al. 2000; Buard and Vergnaud 1994; Jeffreys et al. 1994), or strand slippage (Levinson and Gutman 1987). Most minisatellites have been mapped to telomeric and centromeric regions (Royle et al. 1988) and were proposed to constitute recombination hot spots (Chakravarti et al. 1986; Steinmetz et al. 1987) or fragile sites (Oliva et al. 2000).

Minisatellites are destabilized through strand slippage (Levinson and Guttman 1987) and have been observed from DNA replication component mutants in yeast. Deletion of the *Saccharomyces cerevisiae* (Ascomycota: Saccharomycetales) *rad27* nuclease involved in Okazaki fragment maturation resulted in an 11-fold increase in the rate of minisatellite mutation (Koskoska et al. 1998). A temperature-sensitive mutant *pol3-t* allele from yeast (Tran et al. 1995; Tran et al. 1996) increased the rate of minisatellite instability 13 fold through an altered catalytic subunit of DNA polymerase (Kokoska et al. 1998). Mutation of the yeast DNA replication processivity factor, proliferating cell nuclear antigen (PCNA), encoded by the *POL30* gene was characterized to have defects in DNA replication. Specifically, the cold-sensitive *pol30-52* mutation caused a six-fold increase in observed minisatellite mutations (Kokoska et al. 1999).

Experiments with yeast estimated the rate of GT–CA microsatellite mutation at 6.7×10^{-6}, and a 20-nucleotide repeat unit minisatellite at 7.4×10^{-5} (Ayres Sia et al. 1997). Microsatellite repeat unit changes were shown to arise as neutral mutations in accordance with the hypothesis of random drift (Jeffreys et al. 1988). Multiple allelic types at each locus have been used in the estimation of fungal genetic diversity (Bart-Delabesse et al. 1998; Bart-Delabesse et al. 1999; St. Leger et al. 2000). Minisatellite motifs discovered within fungi have included a 12-bp repeat from the unicellular brewing yeast *Saccharomyces carlsbergensis* (Ascomycota: Saccharomycetales) (Anderson and Nilsson-Tillgren 1997), that was found within a homolog of the *S. cerevisiae* open reading frame (ORF) YCL010c. Two subtelomeric minisatellites, STR-B (Louis et al. 1994) and the Y element (Horowitz and Haber 1984), were found to consist of 36- and 56-bp repeat elements, respectively. A minisatellite from the filamentous ascomycete *Podospora anserina* (Ascomycota: Sordariales) has been characterized (Hamann and Osiewacz 1998). The *P. anserina* locus *PaMin1* consisted of a GT-rich, 16-bp repeat element and intraspecies variation defined six allelic types. The second known minisatellite from a filamentous ascomycete fungus, *MSB1*, was discovered in the *Botrytis cinerea* (Ascomycete: Leotiale, Sclerotiniaceae) ATP-synthase intron and contained seven allelic types that varied in the number of AT-rich, 37-bp repeat motifs (Giraud et al. 1998).

We report *B. bassiana* isolates that were differentiated on the basis of allelic types present at the newly described minisatellite locus, *BbMin1*, that varied in the number of 16 nucleotide repeat units. The minisatellite was only the third such motif to be isolated from a filamentous ascomycete fungus. Interspecific amplification of the locus from four related species, *Beauveria amorpha*, *Beauveria brongniartii*, *Beauveria caledonica*, and *Beauveria vermiconia* was desired in order for evolutionary conservation of the locus to be determined. We wished to use *BbMin1* allele variation to compare isolates in respect to the geography and insect-host preference displayed by the entomopathogenic fungus *B. bassiana*, which may resolve ambiguity between previous studies.

Materials and methods

Beauveria isolates and sample preparation

Sixty-six *B. bassiana* (Bb) isolates and one isolate each of *B. amorpha* (Ba), *B. brongniartii* (Bt), *B. caledonica* (Bc), and *B. vermiconia* (Bv) were obtained from the U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS), Plant Protection Research Unit, U.S. Plant, Soil, and Nutrition Laboratory, Ithaca, N.Y. (Humber 1992). Bb6715 was originally isolated from an adult western corn rootworm, *Diabrotica virgifera* subsp. *virgifera*, and received from Barbra Mulock, USDA–ARS, Brookings, S.D. Bb726 was isolated from a grasshopper, by Stephan Jaronski, Myotech Corp., Butte, Mont. Field isolates EL03 and EL12–EL19 were derived from European corn borer larvae, *Ostrinia nubilalis* (Lepidoptera: Crambidae) and maintained at the USDA–ARS Corn Insects and Crop Genetics Research Unit (CICGRU), Ames, Iowa. Isolates NR1–NR5 were from northern corn rootworm, *D. barberi* (Coleoptera: Chrysomelidae), adults and WR1–WR15 were from *D.*

Fig. 1. Insert DNA sequence 483-bp from clone pGEM-BbMSGA-07 (GenBank accession No. AF387913). Alternate 16-bp 5′-GAGAATATCAGACGGG-3′ repeat units and four GT repeats are underlined, and primer binding sites are underscored by arrowheads, indicating direction.

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001-GAGAGAGAGA GAGAGAGTGA GTGTGGACAC CGAAACACGC CCACAACGCT ATTGACCGAG AACAGCTGTT

071-GATTAAAATC TACGAAATGG CCGGGTACCT ACAGCGCATC TGCAAAGGCC CTGCAGCAGA GCTGCATTTT

141-TTTTTTCTGC CGCTCTTGGC TCCATCTGCC CGAGCTCTGA GGCTCTGACT ATCTTGAATT TGACGGCTTT

211-TACTGCCATC ACCCCAATTA GCAGCCGTAT TTGGTTATGC AGAGTTACGG ACGGCTGCAG CAGCCACATG
                                                                BbMin1-F >>>>
281-TTGGTGACGA AGTGAGCCCA CCACGACCTG CCACCGGTGT GTGTGGGACG TGGGGAGAAT ATCAGACGGG
    >>>>>>>>>> >>>>>>>
351-GAGAATATCA GACGGGGAGA ATATCAGACG GGGAGAATAT CAGACGGGGA AGAATATCAG ACGGGGAGAA

421-TATCAGACGG GGAGAATATC AGACGGGGAA GAATATCAGA CGAGGGCTCT CTCTCTCTCT CTC
                                  <<<<<<<< <<<<<<<<<< <<<<< BbMin1-R
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virgifera subsp. *virgifera* (Coleoptera: Chrysomelidae) adults collected from two fields in Jackson Co., Iowa, approximately 2 km apart. Bb1022 was isolated by the USDA–ARS CICGRU from a corn plant near Champaign, Ill. Propagation was on Sabordauds dextrose agar (Difco, Becton Dickenson Co., Sparks, Md.) incubated at 30°C. DNA extractions were performed as described by Neuveglise et al. (1997). Resultant nucleic acid pellets were diluted with sterile deionized water and stored at –20°C before use.

Satellite DNA isolation

Degenerate $(GA)_8RY$ primers were used to amplify total genomic DNA from isolate Bb1022 in a touchdown PCR (Don et al. 1991). Products were separated on a 0.1 × 20 cm, 6% polyacrylamide, 29:1 (acrylamide:bisacrylamide), 1× Tris-borate–EDTA (TBE) gel with 20 µL PCR product per lane. Allele fragments were visualized by ethidium bromide staining, and image capture took place on a Fotodyne FOTO – Analyst Investigator PC-FOTO – Eclipse Electronic Documentation System (Fotodyne, Hartland, Wis.). PCR product ligation into the pGEM-T Easy cloning vector (Promega) took place according to manufacturers instructions. *Eschericia coli* SURE® (Stratagene, La Jolla, Calif.) was transformed by electroporation on a MicroPulsar apparatus (BioRad, Hercules, Calif.). Clone selection and blue–white screening were performed (Maniatis et al. 1989). Eleven clones with unique insert sizes were propagated in 25 mL terrific broth (12 g tryptone, 24 g yeast extract, 4 mL glycerol, 2.31 g KH_2PO_4, and 12.54 g K_2HPO_4) containing ampicillin (Maniatis et al. 1989). Plasmid DNA was isolated with the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.) according to manufacturer's directions. Template was submitted to the DNA Sequencing and Synthesis Facility at Iowa State University in the concentration of 50 ng/µL. Individual plasmids were sequenced in separate reactions with primers T7 and SP6. Each insert DNA sequence was reconstructed from T7 and SP6 reaction electropherogram data using Contig Express (Informax, San Francisco, Calif.).

PCR amplification and screening

Primers BbMin1-F (5′-CATGTTGGTGACGAAGTGAGC-3′), and BbMin1-R (5′-GAGAGAGAGCCCTCGT CTGATAT-3′) were designed based on insert DNA sequence data from clone pGEM-BbMS-07, and using the Primer3 website (Rozen and Skaletsky 1998). Both oligonucleotides were synthesized at Integrated DNA Technologies (Coralville, Iowa). PCR amplification took place using 6 pmol of each primer, 20–25 ng of sample DNA, 0.425 U *Taq* polymerase (Promega), 1.25 µL of 10× thermal polymerase buffer (Promega), 2.5 mM $MgCl_2$, and 150 µM dNTPs in a 12.5-µL volume. Thermocycler reaction took place at 94°C for 3 min, followed by 40 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. PCR products were separated at 150 V for 5 h on a 20 × 0.1 cm, 6% polyacrylamide, 19:1 (acrylamide:bisacrylamide), 1× TBE gel with a 25-bp ladder (Promega) for size comparison. Bands were visualized by ethidium bromide staining, and image capture took place on a Fotodyne FOTO – Analyst Investigator PC-FOTO – Eclipse Electronic Documentation System (Fotodyne, Hartland, Wis.). DNA fragment size estimations were made from the digital images using Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, Md.).

Estimated repeat number of each allele size was confirmed by sequencing *BbMin1* PCR products from isolates Bb3543, Bb730, Bb1022, Bb938, and Bb201. PCRs described previously were scaled up to 50 µL, and were purified using the QIAprep spin miniprep kit (Qiagen, Valencia, Calif.) according to manufacturer directions. Owing to the difficulty in sequencing small PCR products, *BbMin1* alleles from isolates Bb3167 (87 bp), Bb1155 (103 bp), and Bb726 (119 bp) were cloned into pGEM-T easy cloning vectors (Promega). Alleles inserted into each plasmid clone were identified using colony PCR with primers BbMin1-F and BbMin1-R, followed by electrophoresis as indicated above. Plasmid DNA was prepared for sequencing as described previously.

Data analysis

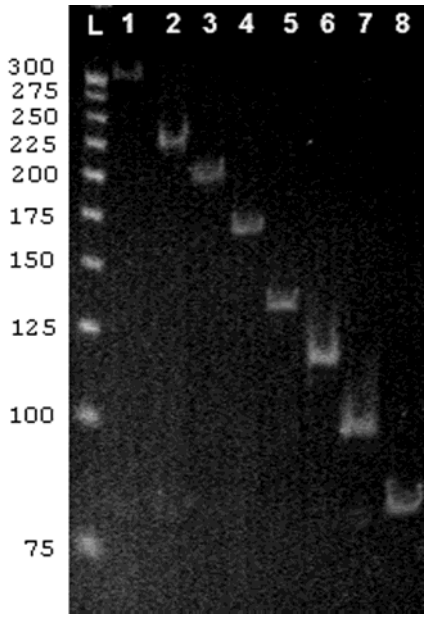
Beauveria bassiana population dynamics were separately analyzed on the basis of pathogenic capacity and geographic distribution. Four ecotypes (subpopulations) were defined on the basis of which insect order isolates were derived (Humber 1992). Each ecotype consisted of two groups that further defined isolate phenotype; Ecotype 1 consisted of two groups, *Ostrinia nubilalis* and other Lepidopteran insects; Ecotype 2 consisted of groups *Diabrotica* spp. and other Coleoptera; Ecotype 3 contained the groups Hemiptera or Homoptera and Hymenoptera; and Ecotype 4 contained groups Orthoptera and all other insects. Five geographic subpopulations were defined from Eastern Asia and Austra-

Table 1. The distribution *BbMin1* minisatellite alleles among 95 *Beauveria bassiana* divided into four ecotypes.

R	Size (bp)	Frequency	Ecotype 1	Ecotype 2	Ecotype 3	Ecotype 4
14	295	0.01	[3543] = N			
10	231	0.01			[730] = S	
08	199	0.253	[501, 502, 533] = A [1149, 1314] = E [1022, 1038, 3113, EL03, EL13-EL15, 2570] = N [959] = S	[1060, 1454, 2520] = S [3037, NR5] = N	[654, 708] = A [2869] = E	[3216] = N [Bt958] = A
6	167	0.07	[2737] = A	[758, 809, 928, 937, 938] = S	[560] = A [1486] = S	
04	135	0.347	[843] = E [1121, EL12, EL16-EL19] = N	[201, 2330, 2579, 3111, NR01-NR04, WR01-WR15] = N	[320] = S	[356] = A [796] = S
03	119	0.05	[151] = E	[150] = E [1155] = F	[477] = S	[1959] = S
02	103	0.221	[652] = A [1113] = E [1001, 2297] = S	[721, 783, 2515] = S [318, 3369, 6715] = N [2685] = F	[300] = A [338]* [886] = E [3086] = N [737, 957, 1960] = S	[726, 1151] = N [Ba2251] = S
01	87	0.03	[2629] = S		[3167] = E	[153, Bc2567] = E

Note: Ecotype 1, Lepidoptera; Ecotype 2, Coleoptera; Ecotype 3, Hemiptera or Homoptera and Hymenoptera; and Ecotype 4, Acrididae and other insects. Within each ecotype, isolates appear in square brackets to indicate geographic location: A, Eastern Asia and Australia; E, Eurasia; F, Africa; N, North America; S, South America; and asterisk, unknown. R indicates repeat number, and size is given in base pairs. Isolates of *B. amorpha* (Ba), *B. brongniartii* (Bt), and *B. caledonica* (Bc) are underlined.

Fig. 2. Polyacrylamide gel electrophoresis separation of eight observed alleles at the *BbMin1* minisatellite locus, performed on 20 × 0.1 cm, 6% polyacrylamide (19:1, acrylamide:bisacrylamide), 1× TBE gels at 150V for 5 h. Alleles with 14 (lane 1), 10 (lane 2), 8 (lane 3), 6 (lane 4), 4 (lane 5), 3 (lane 6), 2 (lane 7), and 1 (lane 8) repeat units are shown. L = Promega 100-bp ladder.



lia (A), Eurasia (E), Africa (F), North America (N), and South America (S). All calculations were performed using Arlequin (Schnieder et al. 1997). Analysis of molecular variance (AMOVA) and θ (F_{st}) were determined by methods described by Excoffier et al. (1992), Weir and Cockerham (1983), and Weir (1996).

Results

A 483-bp plasmid-insert DNA sequence from clone pGEM-BbMS-07 contained a 16-bp minisatellite motif with seven repeats of 5′-GAGAATATCAGACGGG-3′ (Fig. 1; GenBank accession No. AF387913), and was subsequently named *B. bassiana* minisatellite 1 (*BbMin1*). The pGEM-BbMS-07 insert sequence that contained *BbMin1* also had a short internal $(GT)_4$ microsatellite and, by nature of its construction, had two flanking $(GA)_8$ microsatellites. Initial PCR amplification of the locus *BbMin1* with primers BbMin1-F and BbMin1-R took place from isolate Bb1022 DNA, and resulted in a 199-bp product as predicted from the cloned sequence. Ninety-five *B. bassiana* isolates were similarly PCR amplified and showed that *BbMin1* was monoallelic in each haploid isolate and size variable among isolates, with eight alleles from 87 to 295 bp in length (Table1; Fig. 2). The 95 isolates were divided into four ecotypes, based on insect-host range, and five groups, according to geographic location of original isolation (Table 1). The frequency of each allele was calculated for the entire population (Table 1). DNA sequence data from isolates Bb3543, Bb730, Bb1022, Bb938, Bb201, Bb1155, Bb726, and Bb3167 (data not shown), representing all observed *BbMin1* allele size variants, identified full repeat units as the basis for each allelic size variant. The locus was also amplified from related species *B. amorpha*, *B. brongniartii*, and *B. caledonica* (Table 1), but amplification from a single *B. vermiconia* sample failed despite repeated optimization attempts. Population structure and relation among defined *B.*

Table 2. AMOVA table comparing *B. bassiana* from eight groups within four ecotypes that define the insect order from which isolates were derived.

		Insect host range				Geographic origin		
	df	Sum of squares	Variance component	% variation	df	Sum of squares	Variance component	% variation
Among groups	3	55.287	0.55285 *V*a	7.81	4	5.916	0.04659 *V*a	11.37
Among ecotypes within groups	4	26.994	0.02802 *V*b	0.40	12	7.644	0.07297 *V*b	17.8
Within ecotype	86	558.92	6.49909 *V*c	91.8	77	22.641	0.29027 *V*c	70.83
Total	93	641.202	7.07995	100	93	36.200	0.40983	100
	Fixation Indices		95% Confidence intervals		Fixation Indices		95% Confidence intervals	
	F_{sc} (F_{IS})	0.00429	0.98143 + –0.00414		F_{sc}	0.20090	0.81723 + –0.00232	
	F_{st}	0.08204	0.08309 + –0.00899		F_{st}	0.29175	0.15284 + –0.01952	
	F_{ct} (F_{IT})	0.07809	0.21896 + –0.01215		F_{ct}	0.11369	0.18084 + –0.01307	

Note: Ecotype 1 consisted of two groups, *Ostrinia nubilalis* and other lepidopteran insects; Ecotype 2, of *Diabrotica* spp. and other Coleoptera; Ecotype 3, of Hemiptera or Homoptera and Hymenoptera; and Ecotype 4 contained Acrididae and all other insects; df, degrees of freedom.

bassiana ecotypes and geographic divisions were evaluated by AMOVA and fixation indices (Table 2).

Discussion

Allele size differences at the *B. bassiana* minisatellite locus *BbMin1* have been characterized and used for identification of isolates. The locus is the third such repeat element reported from a filamentous fungus, where the first was a GT-rich 16-bp repeat at the *PaMin1* locus of the ascomycete *Podospora anserina* (Hamann and Osiewacz 1998). Like *PaMin1*, *BbMin1* was isolated from a genomic clone that contained a microsatellite. Linkage between microsatellite and minisatellite elements also was reported from human genomic clones (Giraudeau et al. 1999). Each *BbMin1* repeat unit (5′-GAGAATATCAGACGGG-3′) has 50% G/C-content and a reduced core region (underlined) that is similar to a majority of G-rich minisatellites (Dover 1989). Electrophoresis of *BbMin1* PCR products indicated that allelic polymorphism existed among isolates of *B. bassiana*, with eight alleles that contained 1–14 repeat units. Based on *BbMin1* allele differences, a fixation index (F_{st}; θ) of 0.08204 suggested that little genetic divergence had occurred between pathogenic ecotypes. AMOVA indicated that 91.8% of *B. bassiana* population variation was present between individuals within each pathogenic ecotype and 0.4% occurred between ecotypes. Similarly, AMOVA results predicted 70.83% of population variation was within groups separated by geographic location, but greater genetic separation was present compared with pathogenic ecotypes (F_{st}; θ = 0.29175). Therefore, we concluded that little statistical evidence existed to correlate *BbMin1* allele size with either insect host preference or geographic location.

The number of alleles maintained among individuals in a population depends upon the rate of generation by mutation and loss by genetic drift. Rate of minisatellite allele loss is assumed to be constant, and polymorphism is dependent upon the rate of allele generation (Jarman and Wells 1989). The distribution of minisatellite alleles is skewed favoring those of decreased repeat number, and implies greater stability with decreased allele length (Wong et al. 1986). Hamann and Osiewacz (1998) suggested that unequal crossover of complete *PaMin1* repeat units during nuclear division (Jarman and Wells 1989; Tautz and Schlotterer 1994) was responsible for minisatellite generation in *P. anserina*. Rarity of somatic recombination (Buard et al. 2000) would imply that generation of new minisatellite alleles has been an infrequent event. Range of *BbMin1* allele sizes among *B. bassiana* isolates and presence of alleles with large repeat number (isolates Bb3543 and Bb710) may suggest an alternate mechanism has functioned in *BbMin1* repeat expansion. Giraud et al. (1998) proposed strand slippage (Levinson and Guttman 1987) as the mechanism by which minisatellite mutation occurred in the ascomycete *B. cinerea*. Mutation in *S. cerevisiae* DNA replication and repair elements RAD27 (Koskoska et al. 1998), POL3 (Koskoska et al. 1998), and POL30 (Kokoska et al. 1999) have also been implicated in minisatellite allele generation. The mechanism by which repeat number has expanded and contracted at *BbMin1* is yet to be determined, but may have involved one or all processes of somatic recombination, strand slippage, or DNA replication and repair errors.

In total, 62 of 95 *B. bassiana* isolates showed *BbMin1* alleles with 1–4 repeat units, and 31 of 95 isolates showed alleles with 6 or 8 repeat units. The largest allele sizes contained repeat unit numbers of 10 and 14, and each were present in 1 of 95 isolates. Population variation at the *PaMin1* locus demonstrated that *P. anserina* isolates differed by up to four repeat units (Hamann and Osiewacz 1998), and seven alleles at the *MSB1* locus of *B. cinerea* varied between 5 and 11 repeat units (Giraud et al. 1998). The frequency of *BbMin1* alleles with increased repeat number could be evaluated in two ways. First, the polymorphic state of *BbMin1* among *B. bassiana* isolates may be explained if a relatively high rate of new minisatellite allele generation is assumed. Second, the original *BbMin1* repeat unit may have undergone duplication early in evolutionary history and the rate of new *BbMin1* allele generation may have been low owing to the lack of a meiotic process in the *B. bassiana* genome. Therefore, the presence of multiple allelic forms would be

attributable to time since original repeat unit duplication. The second hypothesis may be supported by proposed mechanisms of mutation at other ascomycete minisatellite loci (Hamann and Osiewacz 1998; Giraud et al. 1998) and multiple repeats characterized from related *Beauveria* species (see below).

The *BbMin1* minisatellite locus was PCR amplified from the *Beauveria* species *B. amorpha*, *B. bassiana*, *B. brongniartii*, and *B. caledonica*. Results indicated that flanking DNA sequence and the repeat unit have been evolutionarily conserved. Tandem 16-bp repeat units were characterized from single *B. amorpha* and *B. brongniartii* isolates, whereas a *B. caledonica* isolate retained a single copy of the repeat. Presence of greater than one repeat unit in the genome of three *Beauveria* species suggested that repeat unit duplication occurred before speciation. Loci with variable numbers of tandem repeat units sometimes have gone undetected among close taxonomic relatives (Angers and Bernatchez 1997; Taylor et al. 1999) and failed amplification of *BbMin1* from *B. vermiconia* isolate Bv2922 was another example. *Beauveria vermiconia* was identified as a primitive species because of a lack of an entomopathogenic phenotype (Mugnai et al. 1989). During time since common ancestry with other *Beauveria* species, point mutations at the BbMin1-F or BbMin1-R primer binding sites of *B. vermiconia* may have occurred.

Previous data indicated that *B. bassiana* isolates were similar among those obtained from the same host insect or same geographic region (Magnai et al. 1989; Poprawski et al. 1989; Neuveglise et al. 1994; Cravanzola et al. 1997; Berretta et al. 1998; Piatti et al. 1998). Berretta et al. (1998) suggested that a shared genetic character was associated with isolates that were most virulent toward *D. saccharalis* larvae. Berretta et al. (1998) also indicated that similar PCR–RAPD patterns among isolates of Argentina and Brazil provided evidence for clonal reproduction (St. Leger et al. 1992). Results from *B. brongniartii* rRNA ITS region PCR–RFLP assays indicated isolates from the insect *Hoplochelus marginalis* were genetically identical regardless of their point of origin (Neuveglise et al. 1994), which was interpreted to suggest linkage between genotype and pathogenic phenotype.

Few minisatellite or microsatellite studies have been conducted on pathogenic fungi. An investigation of *Aspergillus flavus* reported that no significant genetic similarity was present among infective types (St. Leger et al. 2000). We suggested that the mutation rate of *BbMin1* was low, and implicated the time since original repeat duplication as the basis for high allelic variability. We further hypothesize that the independent distribution of the *BbMin1* allele among pathogenic types and geographically distant isolates of *B. bassiana* (Table 2) is because of neutral mutation and subsequent random genetic drift (Jeffreys et al. 1988). We identified three subsets of isolates that occupied common ecological niches and were likely to share a recent common ancestry; those isolated from *O. nubilalis* larvae (*i*) near Ames, Iowa; (*ii*) in China; and (*iii*) those from *Diabrotica* spp. adults from North America. Eight *B. bassiana* isolates from *O. nubilalis* larvae from Iowa were collected from an area 15 km^2 and had two *BbMin1* alleles that differed by four repeat units (four mutation steps) (Table 1). Four isolates that infected *O. nubilalis* in China had two separate alleles that were separated by a difference of five repeat units. Twenty-three isolates collected from closely related members of the insect genus *Diabrotica* in North America possessed three different *BbMin1* alleles separated by as many as five repeats. Allelic differentiation between isolates that share similar ecological niches may indicate that *B. bassiana* is more genetically heterogeneous that previously reported (Cravanzola et al. 1997; Berretta et al. 1998; Piatti et al. 1998), and could support the existence of multiple sympatric lineages (Urtz and Rice 1997).

The locus *BbMin1* contained the first minisatellite described in the genus *Beauveria*. The molecular genetic marker was used for identification of isolates from *B. bassiana* and related species *B. amorpha*, *B. brongniartii*, and *B. caledonica*. AMOVA and fixation indices suggested no relation between *BbMin1* allelic component and insect-host preference or geographic origin. Increased rate of mutation at the minisatellite locus *BbMin1* may account for the dissimilarity of alleles among isolates that occupy the same ecological niche and geographical location or those that share a recent common ancestry. Allelic variation at the *BbMin1* locus suggested that it is a neutral genetic marker. Additional satellite DNA markers are being developed to continue investigation of satellite region mutation in ascomycete fungi.

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